INFECTION AND IMMUNITY, Sept. 2005, p. 6064–6074 Vol. 73, No. 9
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.9.6064–6074.2005

Identification of Virulence Determinants for Endocarditis in *Streptococcus sanguinis* by Signature-Tagged Mutagenesis†

Sehmi Paik,[1,2]‡ Lauren Senty,[1,2] Sankar Das,[1] Jody C. Noe,[1,2]§ Cindy L. Munro,[3] and Todd Kitten[1,2,4]*

The Philips Institute of Oral and Craniofacial Molecular Biology,[1] Departments of Microbiology and Immunology[2] and Adult Health Nursing,[3] and the Center for the Study of Biological Complexity,[4] Virginia Commonwealth University, Richmond, Virginia

Received 27 January 2005/Returned for modification 16 March 2005/Accepted 13 May 2005

Streptococcus sanguinis **is a gram-positive, facultative anaerobe and a normal inhabitant of the human oral cavity. It is also one of the most common agents of infective endocarditis, a serious endovascular infection. To identify virulence factors for infective endocarditis, signature-tagged mutagenesis (STM) was applied to the SK36 strain of *S. sanguinis*, whose genome is being sequenced. STM allows the large-scale creation, in vivo screening, and recovery of a series of mutants with altered virulence. Screening of 800 mutants by STM identified 38 putative avirulent and 5 putative hypervirulent mutants. Subsequent molecular analysis of a subset of these mutants identified genes encoding undecaprenol kinase, homoserine kinase, anaerobic ribonucleotide reductase, adenylosuccinate lyase, and a hypothetical protein. Virulence reductions ranging from 2-to 150-fold were confirmed by competitive index assays. One putatively hypervirulent strain with a transposon insertion in an intergenic region was identified, though increased virulence was not confirmed in competitive index assays. All mutants grew comparably to SK36 in aerobic broth culture except for the homoserine kinase mutant. Growth of this mutant was restored by the addition of threonine to the medium. Mutants containing an insertion or in-frame deletion in the anaerobic ribonucleotide reductase gene failed to grow under strictly anaerobic conditions. The results suggest that housekeeping functions such as cell wall synthesis, amino acid and nucleic acid synthesis, and the ability to survive under anaerobic conditions are important virulence factors in *S. sanguinis* endocarditis.**

Infective endocarditis is a life-threatening endovascular infection believed to occur when bacteria in the bloodstream adhere to damaged heart valves. Conditions that cause heart valve damage are risk factors for native valve endocarditis and include congenital heart disease, chronic rheumatic heart disease, and mitral valve prolapse with regurgitation (48). Fibrin and platelets are deposited at the site of endothelial cell trauma, forming a sterile vegetation where bacteria may adhere and colonize during bacteremia. Oral streptococci may enter the bloodstream through invasive dental procedures; therefore, antibiotic prophylaxis is recommended prior to such procedures for at-risk patients (48). It is also clear that some endocarditis cases caused by oral flora are not preceded by dental procedures (48, 59). Thus, other sources of bacteremia, which may include eating or other daily activities (48, 59), must be responsible for these cases. However, antibiotics cannot realistically be used to prevent such occurrences. Therefore, a vaccine would be a preferable prophylactic if one were available.

Several studies have examined putative streptococcal virulence factors for endocarditis to better understand the disease and to identify possible vaccine candidates. Some have implicated binding to laminin, fibrin, and intact extracellular matrix as important in causing endocarditis (48, 62). Platelet aggregation by *Streptococcus sanguinis* has also been shown to contribute to virulence in an animal model (26). Receptors for this interaction have been identified on both bacteria and platelets (6, 31, 55). Other studies have come to conflicting conclusions concerning the exact role of platelets in endocarditis (27, 60). This may be related to the finding that release by activated platelets of microbicidal proteins appears to aid in the clearance of streptococci from vegetations (15, 60). Therefore, successful pathogens may need to activate platelets to cause aggregation but also resist killing by platelet microbicidal proteins (15, 25). A family of related lipoproteins, including FimA from *Streptococcus parasanguinis*, has also been implicated in streptococcal adherence and virulence. A *fimA* mutant of *S. parasanguinis* shows decreased binding to fibrin monolayers in vitro and decreased infectivity in a rat model of endocarditis (9). A FimA ortholog, SloC, is also important for endocarditis causation in *Streptococcus mutans* (34, 53). Both FimA (52) and SloC (53) are members of ABC transport systems that take up manganese and iron, and this function has been shown to be required for endocarditis virulence in *S. mutans* (53). Exopolysaccharide production by *S. mutans*, which contributes to dental caries partly by contributing to adherence, has also been examined in relation to endocarditis. Mutants unable to produce exopolysaccharides adhere less well to fibrin, are less resistant to phagocytosis by human granulocytes in vitro, and show decreased virulence in a rat model

* Corresponding author. Mailing address: The Philips Institute of Oral and Craniofacial Molecular Biology, Virginia Commonwealth University, 521 North 11th Street, Richmond, VA 23298-0566. Phone: (804) 628-7010. Fax: (804) 828-0150. E-mail: tkitten@vcu.edu.

† Supplemental material for this article may be found at http://iai.asm.org/.

‡ Present address: Wayne State University, NIH Perinatology Research Branch, 275 E. Hancock Ave., Detroit, MI 48201.

§ Present address: 2200 Bergquist Drive, Suite 1, 859 MDTS/MTLLM, Lackland AFB, TX 78236-5300.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Source or reference
S. sanguinis strain		
SK36	Human plaque isolate	32
JFP27	*nrdD*Δ1, derived from SK36	This study
Plasmid		
pEMCat	Cm^r, Ap^r; derived from pUC19; contains the 1.4-kb *Himar1*-derived mini-transposon *magellan2*	22
pJFP1	Cm^r; derived from pEMCat by deletion of *bla*	This study
pVA2592	Ap^r, Kan^r; *aphA-3* gene cloned into pUC19	53
pVA2606	Kan^r; suicide vector derived from pUC19 by replacement of *bla* with *aphA-3*	This study
pVA838	Em^r, Cm^r; *E. coli-Streptococcus* shuttle plasmid	41
pJFP29	Em^r; pVA838 containing the 2.7-kb *nrdD* locus	This study
pJFP30	Em^r; pVA838 containing the 2.4-kb *nrdD* locus	This study

of endocarditis (50). Thus, exopolysaccharides likely contribute to adherence and avoidance of immune clearance in *S. mutans*. However, the elimination of exopolysaccharide production in *Streptococcus gordonii* by mutation had no effect on the ability of this organism to cause endocarditis in the same model (66).

Among the viridans streptococci, *S. sanguinis* is most commonly isolated from infective endocarditis patients (17, 58). We therefore chose to examine this species to identify virulence factors that could serve as new targets for drugs or vaccines. The technique of signature-tagged mutagenesis (STM) was used to identify *S. sanguinis* virulence factors for endocarditis in an animal model. STM is a modified transposon mutagenesis system devised by Hensel and colleagues (24). It was originally developed for *Salmonella* species but has been successively applied to many other pathogens (13, 44). STM allows for the large-scale creation and simultaneous in vivo screening of random mutants for alterations in virulence.

For this study, STM was used to create and screen 800 mutants of *S. sanguinis* SK36 in a rabbit model of endocarditis. Two rounds of screening and additional experiments identified six mutants of interest, including strains with mutations in an intergenic region and in genes encoding undecaprenol kinase, homoserine kinase, anaerobic ribonucleotide reductase (RNR), adenylosuccinate lyase, and a hypothetical protein.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2. *S. sanguinis* strain SK36 was obtained from Mogens Kilian (University of Aarhus, Denmark) (32). *S. sanguinis* strains were routinely grown in an atmosphere of 10% H_2, 10% CO_2, and 80% N_2 at 37°C in brain heart infusion (BHI) broth (Difco Inc., Detroit, MI) or on tryptic soy broth (TSB; Difco Inc.) supplemented with 1.5% agar, unless otherwise indicated. Experiments performed in an anaerobic chamber (Forma Scientific, Marietta, Ohio) employed the same atmosphere with a palladium catalyst that maintained oxygen levels at less than 1 ppm. Aerobic growth experiments were performed in 200-μl volumes in microplates using a FLUOstar plate reader (BMG, Offenburg, Germany). Antibiotics for streptococcal selection were chloramphenicol (Cm) at 5 μg/ml and erythromycin (Em) at 10 μg/ml as needed. Todd-Hewitt (TH) broth (Difco Inc.) adjusted to pH 7.6 and containing horse serum (HS) was used for *S. sanguinis* transformation. TH broth was filter sterilized and aliquots stored at −20°C. Working stocks were kept at 4°C for up to a month. Heat-inactivated HS was stored at −20°C, thawed, and added to TH broth to 2.5% immediately before use. *Escherichia coli* DH10B (Invitrogen, Carlsbad, CA) used for cloning was routinely grown at 37°C with shaking in Terrific Broth (63). Cm (5 μg/ml), Em (300 μg/ml), ampicillin (Ap; 100 μg/ml), and kanamycin (Kan; 50 μg/ml) were added as needed for plasmid selection.

Creation of signature-tagged transposons. A pool of signature tags composed of a 40-bp variable central region flanked by 24- and 25-bp invariant ends was provided by David Holden (Hammersmith Hospital, London) (24) and modified to create BglII restriction enzyme sites at either end using primers PB1 and PB2 (Table 3). The plasmid pJFP1 and the signature tags were digested with BglII, ligated to each other, and transformed into *E. coli*. Transformants were selected by Cm resistance.

In vitro transposition. The MarC9 hyperactive form of the *Himar1* transposase was purified from an *E. coli* strain carrying the transposase gene in a pET expression vector as described previously (36). A single purification yielded 17.6

TABLE 2. Properties of selected STM mutants

Mutant	Phenotype	Transposon insertion site best match[a]	% Similarity	Accession no.	CI[b]
3-24	Hypervirulent	Cons. hypoth. {U}	75%	AAT10377	1.6 (0.24–2.9)
		LacR repressor {D}	68%	NP_349552	
4-4	Avirulent	BacA	84%	NP_344977	**0.018** (0.013–0.24)
4-29	Avirulent	ThrB	86%	NP_345818	**0.034** (0.0056–0.061)
6-26	Avirulent	NrdD	93%	NP_357777	**0.31** (0.14–0.47)
9-10	Avirulent	PurB	97%	YP_140478	**0.0063** (0.0024–0.010)
16-29	Avirulent	Cons. hypoth.	72%	ZP_00098115	0.57 (0–1.4)

[a] {U} and {D}, open reading frames immediately upstream and downstream of the insertion, respectively. For all other mutants, the insertion was within the gene indicated. Cons. hypoth., conserved hypothetical protein.

[b] Values are the mean CI values obtained from three or four rabbits each, along with upper and lower 95% confidence intervals. Values in bold are significantly different from 1 ($P < 0.05$).

TABLE 3. Primers used in this study

Primer name	Sequence[a]
PB1	CTAGATCTCTACAACCTCAAGC
PB2	CAAGATCTCCATTCTAACCAAG
PB11	TCTACAACCTCAAGC
PB12	TCCATTCTAACCAAG
Arb1-1	GGCCACGCGTCGACTAGTCANNNNNNNNNNAGCTG
Arb1-2	GGCCACGCGTCGACTAGTCANNNNNNNNNNTGAAC
Arb1-3	GGCCACGCGTCGACTAGTCANNNNNNNNNNTACNG
Arb1-4	GGCCACGCGTCGACTAGTCANNNNNNNNNNCAGCT
Arb1-5	GGCCACGCGTCGACTAGTCANNNNNNNNNNCTGCT
Arb2	GGCCACGCGTCGACTAGTCA
425L19	TTTTCGTTTGTTGAACCAT
M110L14	AGCCCGGGAATCAT
M29L19	AGCGACGCCATCTATGTGT
M1163U18	CCGTTAGTTGAAGAAGGT
M1201U19	TCGGGTATCGCTCTTGAAG
ARTR-SalI-24495-F	GCTTTTGGTCGACAATATCGCAGCCAAG
ARTR-23327-R	AGCCATTCATATGCTTGACTGGAGCAGTTTCAAACTTC
ARTR-21212-F	GTCAAGCATATGAATGGCT
ARTR-SalI-20031-R	TGGTGCGTCGACTGGCTTCGTTGTCTTC
ARTR-EcoI-F1	AAAACAGTCAGCCGAATTCTATTT
ARTR-EcoRI-F2	AGGTTTAAAGAGAATTCATTTGTCCTAAGGG
ARTR-EcoRI-R	TTCGAATTCTGGTCGTCTCAGCTCC
pVA838-2939-F	GTAACACGCCACATCTTG
pVA838-5331-R	CCAATGGCATCGTAAAGAAC

[a] Underlining indicates restriction enzyme recognition sites. N, any of the four nucleotides.

mg of transposase. In vitro transposition reactions were performed with 0.5 μg of donor plasmid (pJFP1 or STM plasmids), 1 to 1.5 μg recipient DNA (SK36 DNA), 200 μg/ml bovine serum albumin, and 2 μg transposase in 20 μl 1× restriction endonuclease buffer 4 (New England Biolabs) at 30°C for 1 h (36, 37). Reactions were stopped by heat inactivation at 70°C for 10 min. Products were treated with T4 DNA polymerase and T4 DNA ligase to fill the gaps generated by transposition and to create intact double-stranded DNA. Each reaction was followed by heat inactivation at 70°C for 10 min. One-eighth of each reaction was used to transform *S. sanguinis* SK36 in the presence of competence stimulating peptide as described below.

Transformation of *S. sanguinis*. The transformation method was based on previously published (21) and unpublished (R. Dwayne Lunsford, SmithKline Beecham Pharmaceuticals, Collegeville, Pennsylvania) methods. Briefly, cultures in 2 ml of TH-HS broth were incubated at 37°C overnight either in an anaerobe jar or with the tube's cap tightly closed. Ten or 50 μl of each culture, respectively, was transferred to 10 ml of TH-HS media similarly preincubated. Incubation was continued at 37°C for 3 to 4 h until the optical density at 660 nm (OD_{660}) reached 0.06 to 0.08. Transforming DNA, 330 μl of cells, and 70 ng of *S. sanguinis* competence stimulating peptide (21) were added to 0.7-ml microfuge tubes, and incubation was continued for 1 h. Cells were plated on tryptic soy agar (TSA) with or without antibiotics and grown in an anaerobe jar at 37°C for 48 h.

STM inoculum preparation. Signature-tagged mutants were grown in individual wells of 48-well blocks in 1.25 ml BHI plus Cm in an anaerobe jar overnight at 37°C. Cells were mixed by vortexing, pooled, and diluted 10-fold into fresh, prewarmed BHI. Incubation was continued at 37°C for 3 h. Cells were harvested by centrifugation at 3,700 × *g* for 10 min at 4°C, washed, and resuspended in phosphate-buffered saline (PBS). Cells were diluted to an OD_{660} of approximately 0.8, equivalent to about 3×10^8 cells per 0.5 ml inoculum.

Animal models. The previously described rat (49, 50) and rabbit (18) models of endocarditis were adapted for this study, employing male Sprague-Dawley rats weighing 200 to 250 g and specific-pathogen-free, male New Zealand White rabbits weighing 3 to 3.5 kg. The protocol received Institutional Animal Care and Use Committee approval (no. 9710-2082 and 0010-2865) and complied with all applicable federal guidelines and institutional policies. In brief, a catheter was inserted through the internal carotid artery of anesthetized animals, past the aortic valve to impose valve damage. The catheter was sutured and remained in the artery throughout the rest of the experiment. Two days later, streptococci were inoculated into the ear vein of catheterized rabbits or the tail vein of catheterized rats. One or two days later, rabbits were sacrificed by intravenous injection of euthasol (Virbac AH, Inc., Fort Worth, TX) and rats were sacrificed by CO_2 inhalation. The heart was removed, and accurate catheter placement was assessed visually. The aortic valve and any apparent vegetations were removed and homogenized with PBS, and multiple dilutions were spread on TSA plates.

Amplification of signature tags and dot blot analysis. Pooled inoculum cells or cells from pooled vegetation homogenate plates containing 5,000 to 50,000 colonies were used for DNA extraction as described previously (53). Signature tags were amplified by PCR using PB1 and PB2 primers (Table 3) and 0.5 to 1 μg DNA. Cycling conditions were 95°C for 1 min; 25 cycles of 94°C for 30 s, 45°C for 60 s, and 72°C for 10 s; and a final 2-min extension at 72°C. Products were purified from 3% NuSieve agarose gels, and 1/10 of each product was used for labeling with a PCR digoxigenin probe synthesis kit (Roche, Mannheim, Germany) and primers PB11 and PB12. The cycling conditions were as described above except that the annealing temperature was 37°C and the number of cycles was 20. Digoxigenin-labeled amplicons were recovered from another 3% NuSieve agarose gel. The gel slice was melted by boiling in hybridization buffer and added directly to a hybridization bottle containing a dot blot membrane. Dot blots were created by transferring 1 μg of each STM plasmid onto a nylon membrane by using a vacuum manifold (Schleicher & Schuell Bioscience, Keene, NH). Signal detection was performed using Genius chemiluminescent kit reagents (Roche) and a Fluorchem Imager with FluorChem software, version 3.04A (Alpha Innotech Corporation, Alexandria, VA).

Identification of interrupted genes by AP-PCR. The arbitrary primed PCR (AP-PCR) technique used was provided by Glen Tamura (University of Washington, Seattle, Washington) and modified during the course of the study (16). The final procedure employed 10 ng of chromosomal template DNA, 0.5 μM arbitrary primer, and 0.2 μM transposon primer for first-round reactions. Cycling conditions were as follows: 95°C for 5 min, followed by six cycles at 95°C for 30 s, 30°C for 30 s, and 72°C for 1.5 min; 30 cycles at 95°C for 30 s, 45°C for 30 s, and 72°C for 2 min; and 72°C for an additional 4 min. PCR products were purified using a QIAquick PCR purification kit (QIAGEN, Valencia, CA). One-eighth of each product was used as template for second-round reactions that employed the Arb2 primer (Table 3) and a nested transposon primer at concentrations of 0.2 μM each. Cycling conditions were as follows: 95°C for 1 min, followed by 30 cycles at 95°C for 30 s, 52°C for 30 s, and 72°C for 2 min, and then 72°C for an additional 4 min. Purified second-round products were used for DNA sequence analysis performed by the Virginia Commonwealth University Nucleic Acids Research Facility with the Arb2 and nested transposon primers.

CI assays. Overnight cultures of SK36 and mutant strains were grown in BHI broth at 37°C. Seven hundred microliters each of the SK36 strain and a mutant culture were coinoculated into a tube containing 12.6 ml prewarmed BHI. Incubation was continued for 3 h. Cells were washed and suspended in PBS to an OD_{660} of 0.8. Five hundred microliters was inoculated into the peripheral ear veins of rabbits. Dilutions of the inoculum were plated using a two-layer plating technique based on a previously published procedure (42). Cells were mixed with 12.5 ml of molten 1.0% low-melting-point (LMP) agarose in TSB at 37°C. The cell suspension was poured into a petri dish, allowed to solidify at room temperature, and incubated for 2 h at 37°C. Molten TSA (12.5 ml at 50°C) with or without 10 μg/ml Cm was then overlaid onto each plate and allowed to solidify. Plates were returned to 37°C and incubated aerobically for 2 days, colonies were counted, and the ratio of mutant to SK36 CFU determined. Catheterization, inoculation, and necropsy were performed as described above. Dilutions of vegetation homogenates were incorporated into layer plates as described above for enumeration. The competitive index (CI) was determined as the mutant/SK36 ratio of the homogenate divided by the mutant/SK36 ratio of the inoculum. Ratios were determined from colony numbers obtained from two to three plates. Since colony numbers are often strongly skewed, the log-transformed values were used. Repeated measures mixed-model analysis of all the colony counts was used to determine CI values, 95% confidence intervals, and whether CI values were significantly different from 1, with $\alpha = 0.05$. All strains were tested in three to four animals each.

Construction of an *nrdD* deletion mutant. Gene splicing by overlap extension (Gene SOEing [28]) was used to construct an internal, in-frame deletion of the *nrdD* gene, fusing the gene's first 12 and last 14 codons. Oligonucleotide pairs ARTR-SalI-24495-F and ARTR-23327-R and ARTR-21212-F and ARTR-SalI-20031-R were used in the first PCR (Table 3). The products of the first reaction served as the template for amplification by primers ARTR-SalI-24495-F and ARTR-SalI-20031-R in a second reaction. The ARTR-24495/20031 product was then cloned into suicide plasmid pVA2606 (Table 1) via SalI restriction sites, creating pJFP27. *E. coli* DH10B was electrotransformed with pJFP27 and plated on Lennox Luria-Bertani agar containing Kan and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Plasmid DNA was prepared using a Quantum

Prep Plasmid Mini-Prep kit (Bio-Rad) and screened by PCR with the ARTR-SalI-24495-F and ARTR-SalI-20031-R primers. DNA sequence analysis confirmed proper insertion and in-frame deletion of *nrdD*. *S. sanguinis* SK36 was transformed with 500 ng SspI-linearized pJFP27 as described above and plated on TSA without antibiotics. Colonies were inoculated into overnight broth cultures for rapid PCR analysis. Briefly, 1 ml of each overnight culture was centrifuged (18,000 × *g* for 2 min) and the pellet was washed in 1 ml 10 mM Tris-Cl, pH 8.0, and suspended in 100 μl 10 mM Tris-Cl. Tubes were then boiled for 5 min, cooled, and centrifuged again, and 100 μl supernatant was transferred to a new tube. Two microliters was used as template for PCR screening reactions employing ARTR-SalI-24495-F and ARTR-SalI-20031-R. Of the 20 colonies screened, 2 produced a band of the expected size for the deleted gene. One of these was selected for further study and named JFP27.

Construction of plasmids for *nrdD* complementation. Two *nrdD* constructs were designed for complementation studies. Primers specific for the two constructs, ARTR-EcoI-F1, ARTR-EcoRI-F2, and ARTR-EcoRI-R (Table 3), were used in PCRs with *S. sanguinis* SK36 genomic DNA as template and with Supermix (Invitrogen). PCR products were purified using Quantum Prep Freeze 'N Squeeze DNA gel extraction spin columns (Bio-Rad) and cloned into the pVA838 (41) shuttle vector via EcoRI restriction sites, creating plasmids pJFP29 and pJFP30 (Table 1). *E. coli* DH10B was electrotransformed with pJFP29 and pJFP30, and transformants were selected on Luria-Bertani agar containing Em. Plasmid DNA was extracted using the Quantum Prep Plasmid Mini-Prep kit (Bio-Rad) and then screened by EcoRI digestion. Sequencing with pVA838-specific primers pVA838-2939-F and pVA838-5331-R (Table 3) confirmed correct construct insertion at splicing junctions. The plasmids were introduced into *S. sanguinis* by transformation with 250 ng of DNA. Transformants were selected by plating on TSA containing Em. PCR with pVA838-specific primers was used to confirm the presence of appropriate plasmids.

Soft-agar study for anaerobic growth sensitivity. For soft-agar studies (61), LMP agarose was dissolved in TSB by autoclaving and aliquoted in 10-ml volumes into glass Hungate tubes. Em and 5 μg/ml of the oxygen dye indicator, resazurin sodium salt (Acros) (5), were added to selected tubes. All tubes were then placed in the 37°C incubator of a Forma Scientific anaerobe chamber for 48 h. Tubes were then inoculated with 100 μl of overnight streptococcal cultures, mixed by inverting, and incubated at ambient temperature in the anaerobe chamber for 30 min to allow the agar to solidify. Tubes were then removed from the anaerobe chamber and incubated aerobically with caps loosened at 37°C for 24 h. The depth of growth was measured in each tube and documented using a digital camera. Values were compared using analysis of variance with the Tukey-Kramer multiple-comparisons test with $\alpha = 0.05$.

Bioinformatics. DNA sequences were viewed, aligned, and edited using SeqMan II software (DNASTAR Inc, Madison, WI). Sequences were searched against the unfinished *S. sanguinis* genome (www.sanguis.mic.vcu.edu/) using the stand-alone version of BLASTN available from National Center for Biotechnology Information (1). GenBank searches were performed using BLASTP. Similarity of paired amino acid sequences was determined by Needleman-Wunsch global alignment using the default parameters of the European Molecular Biology Open Software Suite (EMBOSS) program Needle (57). Oligonucleotide primers were designed using Oligo (Molecular Biology Insights, Cascade, CO) and FastPCR (www.biocenter.helsinki.fi/bi/bare-1_html/fastpcr.htm) software. Multiple sequence comparisons were made using the Genetics Computer Group program Pileup (Wisconsin Package, version 10.3; Accelrys Inc., San Diego, CA) and ClustalW (12). Transcriptional terminators were predicted using the Genetics Computer Group program Terminator (7). DNA sequences of genes identified in the study are available as supplementary material.

RESULTS

Construction of STM mutants in *S. sanguinis*. STM mutants were generated by in vitro transposition using purified *Himar1* transposase and a *mariner*-based minitransposon, *magellan2*. In this system, the purified transposase is sufficient to cause transposition of the minitransposon into the target DNA included in the reaction, although subsequent treatment with polymerase and DNA ligase is required to fill and seal the gaps created by transposition (37). The target for transposition was the genomic DNA of *S. sanguinis* strain SK36. Transposition products were then introduced into cells of the same strain by transformation. The SK36 strain was used for these studies because it was selected for genomic sequencing (Macrina et al., manuscript in preparation) based on a number of properties including adhesion to saliva-coated hydroxyapatite, platelet aggregation, and virulence for endocarditis in a rat model (www.sanguis.mic.vcu.edu/). SK36 was also sensitive to Em, Cm, and Kan.

The pEMCat vector containing the *magellan2* minitransposon (22) was modified by removing the *bla* gene to prevent the possible introduction of β-lactamase resistance into streptococci. The resulting plasmid was designated pJFP1. Pooled signature tags were ligated into the minitransposon of pJFP1 as described by Hava and Camilli (22). Plasmids were screened to select 48 that produced strong signals in blots washed under high stringency conditions. These tagged plasmids were combined into pools for further screening to eliminate cross-hybridizing tags. One plasmid was eliminated. Finally, 40 of the 47 remaining plasmids that contained uniquely hybridizing signature tags, referred to as STM plasmids, were selected. Individual in vitro transposition reactions were performed with each STM plasmid and *S. sanguinis* genomic DNA. A portion of each product was transformed into SK36. Transformants were arrayed in microtiter plates to create sets of 40 uniquely tagged mutants and cryopreserved. In total, 20 sets of 40 mutants each were prepared and tested.

Pilot animal studies. The first set of 40 mutants was inoculated into 18 catheterized rats, which served as a model for endocarditis (49). Inocula of 10^{10}, 10^9, and 10^8 CFU were used. Seven rats were successfully infected: three each from the 10^{10} and 10^8 inocula and one from the 10^9 inoculum. This infection rate is typical for viridans streptococci in this model (9, 34, 53). Dot blot analysis of the inoculum pool and the bacteria recovered from the infected vegetations (output pool) was performed essentially as described previously (22). The output pool of bacteria was recovered from rats 2 days postinfection by spreading heart valve homogenates on agar plates. Replicate dot blots containing 41 STM plasmids were prepared on nylon membranes. Each blot was hybridized with labeled signature tags derived from the pooled inoculum or from the bacteria recovered from each infected vegetation sample. The 41st tagged plasmid served as a negative control since this plasmid was not used for mutant creation. The number of mutant strains that infected each animal ranged from 2 to 27, with a median value of 9. The results from three rats are indicated in Fig. 1A. As shown, strain recovery was inconsistent. This suggested that a colonization bottleneck had occurred (44) so that failure to recover most strains from a vegetation sample was probably the result of chance rather than reduced virulence. Therefore, additional trials were performed using the rabbit endocarditis model. Three inoculum levels were used: 2×10^8, 2×10^9, and 1×10^{10}. The largest inoculum sample proved lethal within 24 h, but there was no apparent difference in the results from the other two inocula. All three inocula produced infections in all animals, and dot blotting revealed consistent results from each animal. An inoculum size of 2×10^8 to 4×10^8 was used for the remaining STM trials. In addition, animals were sacrificed 1 day postinoculation rather than 2.

Screening and selection of STM mutants. Each STM mutant pool was inoculated into two or three rabbits. Figure 1B shows the results of analyzing in rabbits the same mutant pool that

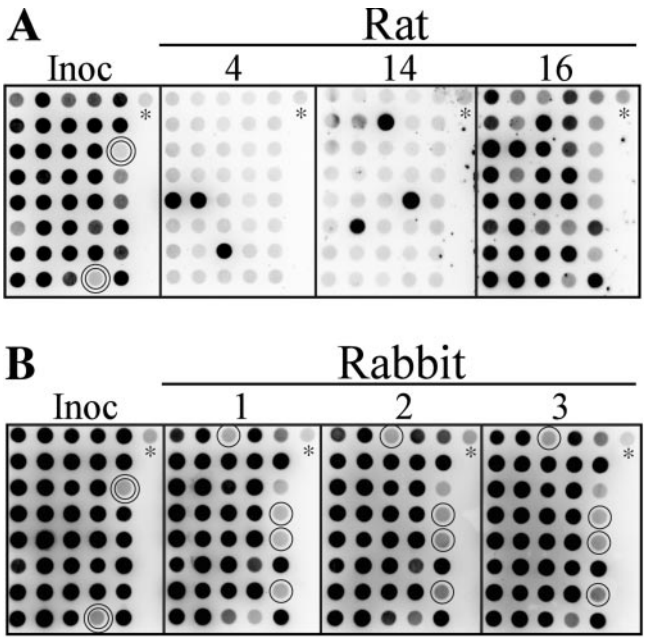

FIG. 1. Dot blot STM analysis. Amplified signature tags from an inoculum of 40 strains (Inoc) and from bacteria recovered from the heart valves of three infected animals were used to probe replicate dot blots. Targets for hybridization were the 40 plasmids bearing the tagged transposons used to create the mutants and a 41st tagged plasmid that serves as a negative control (indicated by an asterisk). (A) Inoculum blot and representative output blots from three rats. (B) Inoculum blot and output blots from three rabbits. Single circles indicate tags that were not detected in the output blots from any of the three rabbits. Double circles indicate tags that were not detected in any inoculum or output blots. The two experiments were performed on separate occasions but employed the same inoculum strains.

was analyzed in rats in Fig. 1A. If a mutant exhibited a hybridization signal comparable to that of the negative control in all output blots but not in the inoculum blot, the strain was identified as a putative avirulent mutant. If a mutant exhibited a strong signal in all output blots but a weak inoculum blot signal, the strain was identified as a putative hypervirulent strain. Strains that produced weak signals in both blots (indicated by double circles in Fig. 1) could not be assessed for virulence. In contrast to our results with rats, most experiments resembled that shown in Fig. 1B in that output pools obtained from different rabbits receiving the same inoculum were comparable.

Primary screening was performed with 800 STM mutants, identifying 53 strains with apparent decreased virulence and 7 with apparent increased virulence. Of these 60 strains, 49 were repooled into three secondary inocula for retesting. STM mutants that appeared to have normal virulence in the initial screen were used to supplement the secondary inocula so that 40 strains would again be tested in each experiment. The secondary screening eliminated 6 mutants, leaving 38 putative avirulent and 5 hypervirulent mutants.

Identification of interrupted genes. Sequences flanking transposon insertions were identified by AP-PCR (16). These sequences were compared to the unfinished SK36 genomic sequence (www.sanguis.mic.vcu.edu/). Analysis of the first four mutants examined by AP-PCR, those corresponding to the circles in Fig. 1B, produced surprising results. These mutants appeared to have vector DNA inserted along with the transposon. Furthermore, chromosomal DNA sequences immediately flanking the insertion were apparently not contiguous prior to mutagenesis (51). Therefore, restriction-digested DNA from remaining mutants was examined by Southern blotting with the pJFP1 plasmid as a probe to identify mutants containing vector sequences. Of the 32 mutants so examined, 12 were eliminated by this screen. Ten additional strains were eliminated because sequencing data showed the presence of the pJPF1 vector sequence adjacent to the minitransposon or because the right and left flanking sequences were noncontiguous. Four other strains either did not generate bands in AP-PCR or were not analyzed. Finally, six mutants with the expected structures, 3-24, 4-4, 4-29, 6-26, 9-10, and 16-29, were collected for further analysis (Table 2). In all mutants except 3-24, a single gene was disrupted by the transposon insertion. In the 3-24 mutant, the transposon was located in a 182-bp intergenic region. In the 4-4 mutant, there was a 48-bp duplication at the site of transposon insertion whereas the other five mutants contained a TA dinucleotide duplication (data not shown). While TA duplication was observed most often upon insertion of the parent transposon of *magellan2*, longer duplications have been observed (37).

Analysis of STM mutant growth and virulence. Production of a strong signal in inoculum blots by five of these six mutants suggested that they likely grew to comparable numbers in the inoculum and were thus well represented there. To examine this possibility more closely, the six mutant strains and SK36 cells were grown in BHI broth in a 96-well plate. Mutant 4-29 grew more slowly than SK36, whereas the other five mutants grew indistinguishably from SK36 and each other (Fig. 2A), suggesting that any growth defects in these five mutants occurred in the animals but not in the broth culture prior to inoculation.

The final six mutants were examined for altered virulence using a CI assay in the rabbit model. SK36 and each mutant strain were coinoculated into catheterized rabbits. The CI was calculated as the ratio of mutant to SK36 in the cells recovered from the infected vegetation samples divided by the ratio of mutant to SK36 in the inoculum. Ratios were determined by plating dilutions of cells with and without Cm. Because we found that Cm^r strains were recovered with reduced and variable efficiency when spread on the surfaces of plates containing Cm, we used a two-layer plating technique in which cells were embedded in LMP agarose and incubated for 2 h at 37°C prior to adding a top layer with or without Cm. With this technique, Cm^r strains were recovered with equal efficiency on plates with and without Cm. The CI results are shown in Table 2. A CI of >1 indicates increased virulence whereas a CI of <1 indicates reduced virulence.

Characterization of individual mutants. Strain 3-24 was identified as a putatively hypervirulent mutant. This was the only mutant in which the transposon insertion did not interrupt a gene. Instead, the insertion was located between genes with similarity to a putative lactose repressor from *Clostridium acetobutylicum* (and other bacteria) and a conserved hypothetical protein from *Streptococcus agalactiae*, with characteristics of an isoprenylcysteine carboxyl methyltransferase (Table 2). The CI

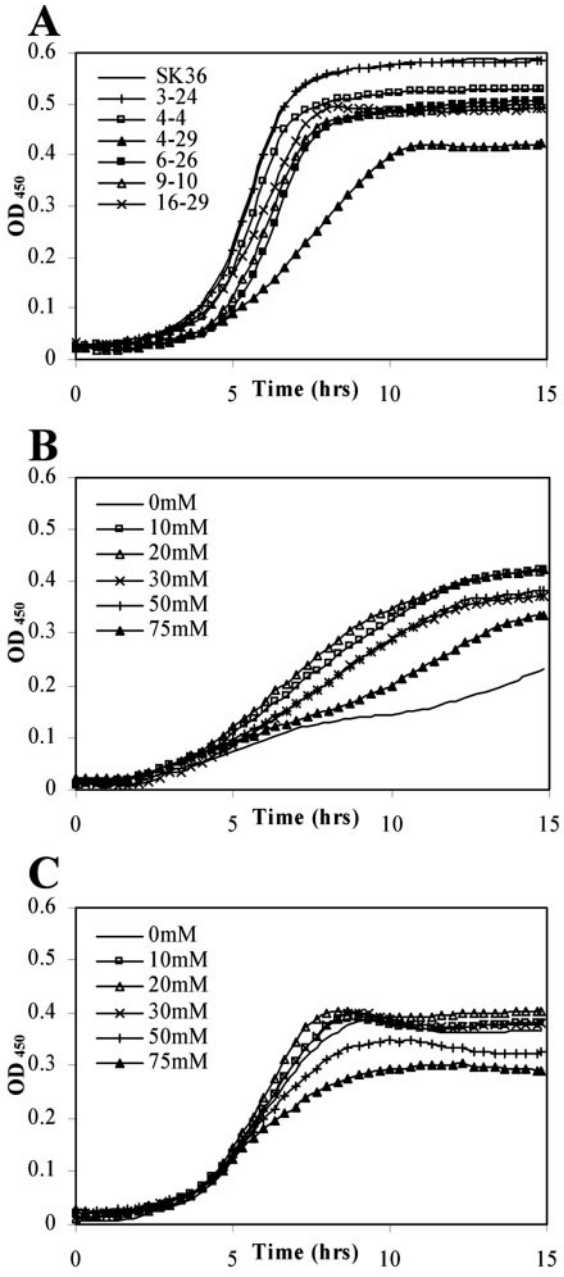

FIG. 2. Growth of STM and SK36 mutants. Cells were incubated at 37°C in a 96-well plate under aerobic conditions, and OD_{450} readings were taken every 20 min. The average absorbance with background subtracted from four or five replicate samples is shown. Experiments were performed on at least two occasions with similar results. (A) Growth of the indicated strains in BHI broth. Growth of strains 4-29 (B) and SK36 (C) in BHI broth containing the indicated concentration of threonine is shown.

assays suggested that 3-24 was moderately more virulent than SK36, though the difference was not significant (Table 2).

Two strains, 9-10 and 16-29, were hypothesized to be defective in nucleotide synthesis. The *purB* gene, encoding adenylosuccinate lyase and mutated in strain 9-10, is involved in de novo purine synthesis (23). The CI value of 9-10 indicated a 150-fold virulence reduction (Table 2). In the 16-29 mutant,

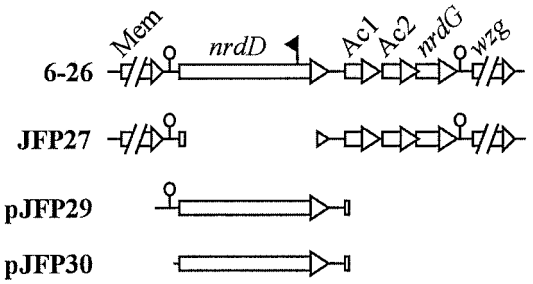

FIG. 3. The *nrdD* locus of *S. sanguinis*. The structure of the *nrdD* locus is indicated for the strains and plasmids listed on the left. Potential terminators are indicated as a circle above a vertical line. The position and orientation of the transposon in strain 6-26 are indicated by the flag. Gene designations are as follows: Mem, putative membrane protein; *nrdD*, anaerobic ribonucleotide reductase gene; Ac1, putative acetyltransferase gene; Ac2, second putative acetyltransferase gene; *nrdG*, anaerobic ribonucleotide reductase small subunit gene; *wzg*, ortholog of putative transcriptional regulator *wzg* of *S. gordonii*.

the disrupted gene is homologous to a conserved hypothetical protein from *Desulfitobacterium hafniense* and other bacteria (Table 2). This protein shows homology to the *fic* gene in *E. coli*, a mutant of which was not able to grow without ρ-aminobenzoate or folate. This suggests that the *fic* gene product may be involved in the synthesis of *p*-aminobenzoic acid (PABA) or folic acid (35). These compounds are required for purine and pyrimidine synthesis in bacteria. The twofold virulence reduction demonstrated by this mutant in CI assays was not statistically significant.

Mutant 4-4 was found to contain a disrupted gene encoding an ortholog of BacA from *Streptococcus pneumoniae* and other bacteria (Table 2). This gene's name was derived from the finding that the *E. coli* ortholog increased bacitracin resistance when expressed from a multiple-copy plasmid (10). Mutation of the *bacA* gene in *S. pneumoniae* and *Staphylococcus aureus* confers bacitracin sensitivity (11). Mutant 4-4 was found to exhibit sensitivity to bacitracin similar to that of *Streptococcus pyogenes* in liquid MIC assays and in disk diffusion assays. *S. pyogenes* is a prototypical bacitracin-sensitive species. Neither SK36 nor another unrelated STM mutant (6-26) exhibited sensitivity (data not shown).

The transposon insertion in mutant 4-29 was in an ortholog of the *S. pneumoniae thrB* gene, encoding homoserine kinase (Table 2). This enzyme is required for the synthesis of threonine (64), and its inactivation has been shown to result in threonine auxotrophy in many bacteria and yeast (67). To confirm the gene's function in *S. sanguinis*, growth studies were performed in BHI supplemented with threonine at 10, 20, 30, 50, and 75 mM. All five concentrations improved the growth of mutant 4-29, with the 75 mM supplementation showing the least effect (Fig. 2B). In contrast, the effect of supplemental threonine on the growth of the SK36 cells was minimal (Fig. 2C). Moreover, the addition of threonine to 50 or 75 mM inhibited SK36 growth. The results thus suggest that the *thrB* gene of *S. sanguinis* was properly identified.

Characterization of a putative anaerobic RNR mutant. In the 6-26 mutant, the minitransposon was inserted in a gene with similarity to *nrdD* of *S. pneumoniae* and other bacteria (Table 2), encoding anaerobic RNR (Fig. 3). Anaerobic, or

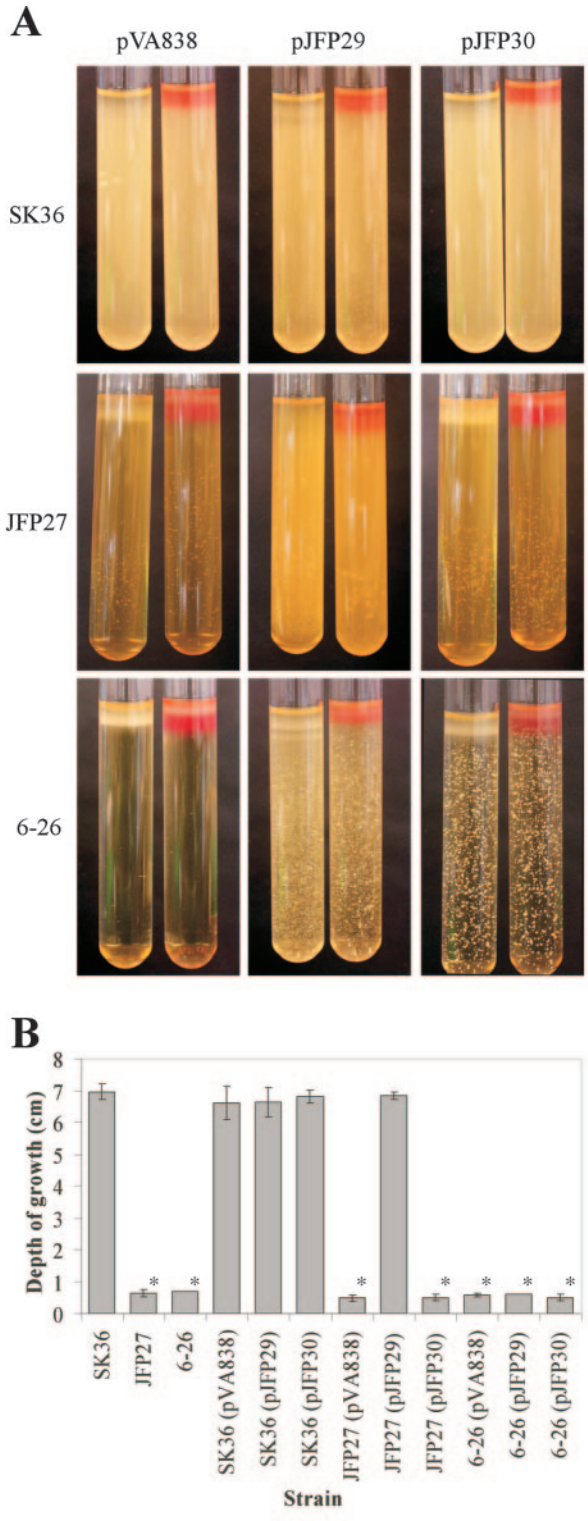

FIG. 4. Growth of *S. sanguinis* strains in an oxygen gradient. The strains indicated were inoculated into anaerobic soft-agar tubes and then incubated overnight with exposure to ambient air to create an oxygen gradient. (A) Photographs of tubes after overnight growth. The strains indicated on the left containing vector alone (pVA838) or vector plus *nrdD* constructs (pJFP29 and pJFP30) were inoculated into tubes with or without the oxygen indicator dye resazurin (right and left tubes in each pair, respectively). (B) Depth of growth in tubes without resazurin. Values are averages ± standard deviations for three samples obtained on at least two separate occasions. Asterisks indicate results significantly different from those for SK36 ($P < 0.001$).

class III, RNRs become permanently inactivated by oxygen (30). Therefore, it was hypothesized that the 6-26 strain would survive only in aerobic conditions. The growth study showed that 6-26 cells grew indistinguishably from SK36 under aerobic conditions (Fig. 2A). Initial studies failed to demonstrate a growth defect using our standard procedure for producing an anaerobic atmosphere.

To test for the possibility that the 6-26 mutant might have acquired an extragenic suppressor mutation to allow anaerobic growth, as well as to eliminate the possible polar effects of transposon insertion, we created an in-frame deletion of the *nrdD* gene in SK36. DNA encoding 705 amino acids was removed, resulting in the fusion of the first 12 codons with the last 14, creating strain JFP27 (Fig. 3). This mutant also grew well on plates in anaerobe jars. A soft-agar oxygen gradient assay (61) employing an anaerobe chamber was therefore used to compare the oxygen requirements of 6-26, JFP27, and SK36. As indicated in Fig. 4, parental strain SK36 grew throughout each tube (typically 7.0 cm in depth), indicating growth in both oxidized and reduced agar. In contrast, 6-26 and JFP27 growth was restricted to oxidized agar, indicated by the pink hue of the oxidized resazurin (Fig. 4 and data not shown). This suggests sensitivity of 6-26 and JFP27 to anaerobic conditions due to the loss of NrdD.

To confirm this, we attempted to complement *nrdD* mutant strains with the *nrdD* gene expressed from its native promoter. Plasmids pJFP29 and pJFP30 contained *nrdD* preceded by 337 or 36 nucleotides, respectively, cloned into the shuttle vector pVA838 (Fig. 3). Sequence analysis predicted a possible terminator 149 bp upstream of *nrdD* (Fig. 3). Plasmid pJFP29 was designed to include the possible terminator and the postulated subsequent native promoter. To exclude the possibility of the *nrdD* gene being expressed from a plasmid-borne promoter, pJFP30 was created, in which the *nrdD* gene was preceded by 36 bp—enough to contain a ribosome binding site but not a complete promoter (Fig. 3).

S. sanguinis SK36, 6-26, and JFP27 were each transformed with pVA838, pJFP29, and pJFP30, and evidence of NrdD complementation was evaluated in soft-agar studies. SK36 growth was unaffected by transformation with pVA838, pJFP29, or pJFP30 (Fig. 4). The *nrdD* mutant strain JFP27 containing pJFP29 exhibited growth that was indistinguishable from that of parent strain SK36. In contrast, pJFP30 did not complement the growth of JFP27, suggesting that *nrdD* in pJFP29 was expressed from its native promoter. Typical dense growth in reduced agar was not restored in strain 6-26 by either pJFP29 or pJFP30. The soft-agar growth of 6-26 (pJFP29) and 6-26 (pJFP30) was distinctive, however, in that isolated colonies were observed growing in reduced agar (Fig. 4A). This phenotype was not exhibited by mutant 6-26 (pVA838). (Smaller spots apparent in some of the other tubes are bubbles in the agar rather than colonies. These were easily distinguished by the lenticular disk morphology of the colonies.)

PCR analysis with chromosome-specific primers (ARTR-

SalI-24495-F and ARTR-SalI-20031-R) was used to assess the genomic *nrdD* region in selected 6-26 (pJFP29) and 6-26 (pJFP30) colonies isolated from reduced agar. Analysis of the PCR products by gel electrophoresis indicated that the transposon had been lost from the genomic *nrdD* in the 6-26 (pJFP30) cells (data not shown). In contrast, the size of the 6-26 (pJFP29) product indicated that the transposon was still present in the *nrdD* gene in these cells. When overnight cultures derived from the characterized colonies were regrown in soft agar as described above, dense growth was observed for both 6-26 (pJFP29) and 6-26 (pJFP30) throughout the tubes, indicating that the capacity for anaerobic growth had been restored in these cells (data not shown).

DISCUSSION

Despite advances in treatment, infective endocarditis mortality remains high, indicating a need for better therapeutic or prophylactic agents. The STM technique was adapted for *S. sanguinis* in the hope of identifying virulence factors for infective endocarditis that could serve as targets for these agents. Initially, 800 STM mutants were screened through two rounds of infection and dot blot analysis procedures using a rabbit model for endocarditis. After the primary and secondary screening, 37 avirulent and 5 hypervirulent strains were identified. Thirty-seven avirulent strains represent 4.6% of the total mutants screened in this study. In previous STM studies employing *S. pneumoniae* in a mouse pneumonia model, the percentage of avirulent strains ranged from 7 to 21% (22, 38, 56), although other analyses have typically produced smaller percentages (44). Coulter et al. (14) performed primary screens of *S. aureus* STM mutants in sepsis, abscess, and wound models and then examined six selected mutants for virulence in an endocarditis model. We are unaware of any study other than ours to perform a primary screen in an endocarditis model.

Our first STM mutant trial employed the rat endocarditis model. As in previous studies (9, 34, 53), over half of the rats inoculated did not develop infections. The rats that were infected yielded 10^5 to 10^8 CFU (data not shown). This suggested a possible strict dichotomy such that rats developed either no infection or heavy infections. The STM results shown in Fig. 1A, however, suggest that most infections were initiated by fewer than 40 bacteria. Surprisingly, there was no correlation between either the number of bacteria or the number of different strains recovered from a given rat and the inoculum size within the range tested. In contrast to the rat model, all 91 of the rabbits employed in this study for STM and CI assays became infected, with a mean recovery of 1.2×10^8 CFU per animal. Nevertheless, seven of these rabbits were infected with only 10^5 to 10^6 CFU. Output blots from six of the seven rabbits were missing many spots, representing STM mutants, that were present in the output blots from other rabbits infected with the same inocula. This suggests that a colonization bottleneck occasionally occurred in the rabbit model as well. Moreover, the mutants that did not infect the rabbits in the first inoculum also did not infect any of the rats (Fig. 1 and data not shown). This suggests that the rabbit model is more permissive for infection than the rat but that the virulence factors required for the infection of both species are similar.

Unexpectedly, the process of mutant creation was more problematic than mutant screening. It is not clear why most of the avirulent mutants we identified had undergone complex recombination events since this was not observed when the same transposition system was used for STM analysis of *S. pneumoniae* (22; A. Camilli, personal communication). Two mutants that did not produce signals in any of the inoculum or output blots (indicated by the double circles in Fig. 1) were found to have had their signature tags deleted (data not shown). Other mutants, such as those circled in Fig. 1, were found to have undergone recombination events that were sufficiently complex to preclude their characterization. We have not yet clarified the mechanism of recombination that led to these mutants; however, subsequent experiments in which individual ~2.5-kb amplicons of SK36 genomic DNA were used as targets for transposition produced aberrant mutants at frequencies of 0 to 100% in side-by-side reactions. This suggests either that the sequence of certain regions causes more aberrant mutants to be formed or that there is selection for aberrant mutants in some genes, perhaps because a conventional mutation would be lethal. The latter possibility is consistent with our findings that both aberrant mutants in which this was examined possessed wild-type copies of the genes surrounding the transposon in addition to the interrupted copies (51).

Despite the high rate of aberrant mutant creation, several strains that had simple transposon insertions were recovered. Strain 3-24 was identified as potentially hypervirulent, since it showed a weak signal in an inoculum blot paired with strong signals in output blots. Four additional uncharacterized mutants also appeared to have increased virulence. This is perhaps not surprising. Competition for the survival of the viridans streptococci occurs in the oral cavity, and there is likely no evolutionary pressure to maintain virulence for endocarditis per se. Moreover, in *S. aureus*, disruption of at least two genes has been shown to cause increased virulence for endocarditis (3, 47).

The 4-4 mutant with a disrupted *bacA* gene exhibited a 55-fold reduction in virulence. Bacitracin acts by binding to undecaprenyl diphosphate, which is the lipid carrier for peptidoglycan and teichoic acid synthesis (2). This binding prevents dephosphorylation to undecaprenol monophosphate, which is required for recycling of the lipid carrier for successive rounds of cell wall synthesis (2). The *bacA* gene product, undecaprenol kinase, is a membrane-bound isoprenol kinase that can phosphorylate cytoplasmic undecaprenol monophosphate, which overcomes the inhibition caused by bacitracin (10). In *S. pneumoniae*, a *bacA* mutant showed markedly reduced virulence in a mouse respiratory tract infection model, though the reason for virulence reduction was not determined (11). The gene was identified as a virulence factor in a later STM analysis of the same organism (38). In another close relative of *S. sanguinis*, *S. gordonii*, a *bacA* mutant was shown to be defective in biofilm formation (40). This study modeled the oral biofilm of dental plaque, but our findings suggest the possibility that *bacA* is also required for formation of the biofilm that occurs in infected vegetations (54).

The *thrB* gene encoding homoserine kinase was disrupted in the 4-29 mutant. This enzyme catalyzes the first committed step for synthesis of threonine and isoleucine (4). Both are essential amino acids for humans who do not possess a homoserine kinase. Therefore, this enzyme has been proposed as a

new antibiotic target (4). The *thrB* gene was identified as a virulence factor in two independent STM analyses of *S. aureus* (14, 45), while *thrC*, also involved in threonine synthesis, was identified in *S. pneumoniae* (38). Purine biosynthesis, which was disrupted by an insertion in the *purB* gene in mutant 9-10, has also been commonly identified in STM studies. The *purB* gene was identified in *S. pneumoniae* (38) and *Streptococcus iniae* (46) STM studies, while *purL* was identified in three *S. pneumoniae* (22, 38, 56) and two *S. aureus* (14, 45) STM studies.

Mutant 6-26 has a transposon insertion in the gene encoding a class III, or anaerobic, RNR—another enzyme that is not present in humans. RNRs are essential for de novo deoxyribonucleotide synthesis (30). Analysis of the unfinished genome suggests that *S. sanguinis* possesses RNRs of classes Ib and III, which is also true for related bacteria such as *S. aureus* and *S. pyogenes* (43). Class I RNRs require O_2 for their activity, whereas class III enzymes are permanently inactivated by O_2 (30). Masalha et al. (43) examined both enzymes in *S. aureus* and found that anaerobic RNR mutants grew more slowly than wild-type *S. aureus* cells under anaerobic conditions, though growth was not completely eliminated. It was suggested that growth in these mutants was due to trace amounts of oxygen in the medium, since addition of the class I RNR inhibitor, hydroxyurea, further reduced growth in these cultures. We also found it difficult to eliminate the growth of *S. sanguinis* RNR mutants under standard anaerobic conditions, suggesting that the class Ib RNR requires only trace amounts of oxygen to support growth. RNR genes have not been identified in STM studies employing streptococci or staphylococci, though a recent STM analysis of *Proteus mirabilis* identified an *nrdD* ortholog as important for virulence in a urinary tract infection model (8).

Oxygen levels in vegetations have not to our knowledge been measured. Coulter et al. (14) determined that five of five unidentified anaerobic *S. aureus* mutants were attenuated for virulence in the rabbit endocarditis model, suggesting that the interior of the vegetation becomes anaerobic despite the oxygenated blood flowing past. The moderate but significant reduction of virulence seen for the 6-26 mutant could suggest either a spatial or temporal gradient of oxygen within the vegetation or a partial ability to overcome the loss of anaerobic RNR activity. Some *Bacillus* species that apparently possess only class I RNRs can use external DNA as a source of deoxyribonucleotides under anaerobic conditions, thereby overcoming the requirement for anaerobic ribonucleotide reduction (19). Given that *S. sanguinis* is also naturally competent and that the biofilm of the vegetation may represent a site of increased natural competence (39), DNA uptake may have aided the survival of the RNR mutants in the CI assays.

Our studies also suggest a model for the transcriptional organization of the *nrdD* locus in *S. sanguinis*. Examination of the incomplete genomic sequence of *S. sanguinis* suggests that the *nrdD* gene is preceded upstream by a putative membrane protein gene and downstream by two acetyltransferase genes and *nrdG*, encoding the beta subunit of the RNR holoenzyme (30) (Fig. 3). The failure of pJFP29 to complement the *nrdD* mutation in strain 6-26 suggests that the transposon mutation was polar on the expression of downstream genes. Complementation of the in-frame *nrdD* deletion mutant JFP27 by pJFP29 but not pJFP30 suggests that the *nrdD* promoter is contained within pJFP29. These conclusions suggest an explanation for the colonies observed in anaerobic agar in some strains (Fig. 4). It appears that in 6-26 (pJFP30), occasional recombination between the chromosomal and plasmid-borne *nrdD* genes caused restoration of the genomic *nrdD* gene and transcription of the remainder of the operon, resulting in colony formation in reduced agar. This likely was not observed in JFP27 (pJFP30) because of the limited sequence shared by the genomic and plasmid-borne loci (72 and 260 bp at the 5′ and 3′ ends of *nrdD*, respectively) (Fig. 3). The retention of the transposon in the genomic *nrdD* gene in colonies of mutant 6-26 (pJFP29) suggests that when *nrdD* is expressed from the pJFP29 plasmid, restoration of growth results from an uncharacterized mutation that restores expression of the chromosomal genes downstream from *nrdD*. Further studies will be needed to confirm these hypotheses. The regions preceding *nrdD* from *S. sanguinis* SK36, two strains each of *S. pyogenes* and *S. pneumoniae*, *S. mutans*, and *S. agalactiae* were compared (see Fig. 2 in the supplemental material). With the exception of SK36, all the species shared potential −35, −16, and −10 promoter sequence elements (65) and other potential promoter elements in the 59 bp upstream of *nrdD*. A homologous promoter region was not found in SK36, suggesting that a unique promoter may regulate *nrdD* expression in *S. sanguinis*.

These findings indicate that housekeeping functions such as cell wall synthesis, amino acid and nucleic acid synthesis, and the ability of the bacteria to survive in anaerobic conditions are important virulence factors for *S. sanguinis* endocarditis. Given that most of the enzymes involved in these processes are not found in humans, this study suggests several possible new drug targets. The finding that the *bacA*, *thrB*, and *purB* genes identified here have also been identified by STM in models ranging from pneumonia in mice (22, 38, 56) to systemic infections in zebra fish (46) suggests that they have a general role in virulence in gram-positive cocci.

There are two groups of genes not identified in this screen that we might have expected to find based on previous studies. The first is composed of the adhesins mentioned in the introduction as having been shown or suspected to be important for endocarditis. One possible explanation for this is the limitation inherent in STM, that certain mutants may be complemented by the other strains in the inoculum, thus masking their virulence defects (24). Indeed, few adhesins have been identified in previous streptococcal STM screens and this may be the reason (22, 29, 38, 46, 56). The second group is composed of those genes identified as induced in *S. gordonii* in the rabbit model of endocarditis, employing in vivo expression technology (33). This could be explained by the observed tendency for this technique to identify genes different from those identified by STM (13). The failure to find either class of genes in our screen could also simply result from the analysis of too few mutants. We recently created a mutation in the *ssaB* gene (20), which is an ortholog of the *fimA* and *sloC* genes required for endocarditis virulence in other viridans streptococci (9, 53). This mutant appeared avirulent by STM and produced a value of 0.00016 in a competitive index assay (Das et al., unpublished data). This suggests that other *S. sanguinis* virulence genes remain to be identified. It also suggests the feasibility of using STM to test strains with targeted mutations in suspected vir-

ulence factors to determine their role in endocarditis virulence. We are currently pursuing this approach.

ACKNOWLEDGMENTS

We thank A. D. Baughn and M. H. Malamy of Tufts University and J. P. Lewis and F. L. Macrina of Virginia Commonwealth University for helpful conversations regarding anaerobic methodology, M. J. Climo for assistance initiating the rabbit endocarditis model, Taisei Kanamoto for assistance with CI experiments, Sarah Maziarz for the initial characterization of mutant 4-4, Al Best for statistical analyses, and Nicai Zollar for technical assistance.

This work was supported by grants 9807751U from the American Heart Association and R01AI47841 and K02AI054908 from the National Institutes of Health to T.K. and by a scholarship from the United States Air Force Institute of Technology to J.C.N.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Anderson, R. G., H. Hussey, and J. Baddiley.** 1972. The mechanism of wall synthesis in bacteria. The organization of enzymes and isoprenoid phosphates in the membrane. Biochem. J. **127:**11–25.
3. **Baddour, L. M., C. Lowrance, A. Albus, J. H. Lowrance, S. K. Anderson, and J. C. Lee.** 1992. *Staphylococcus aureus* microcapsule expression attenuates bacterial virulence in a rat model of experimental endocarditis. J. Infect. Dis. **165:**749–753.
4. **Bareich, D., K. Koteva, I. Nazi, and G. D. Wright.** 2004. Small molecule functional discrimination of the kinases required for the microbial synthesis of threonine and isoleucine. Bioorg. Med. Chem. **12:**807–815.
5. **Behbehani, M. J., H. V. Jordan, and D. L. Santoro.** 1982. Simple and convenient method for culturing anaerobic bacteria. Appl. Environ. Microbiol. **43:**255–256.
6. **Bensing, B. A., J. A. López, and P. M. Sullam.** 2004. The *Streptococcus gordonii* surface proteins GspB and Hsa mediate binding to sialylated carbohydrate epitopes on the platelet membrane glycoprotein Ibα. Infect. Immun. **72:**6528–6537.
7. **Brendel, V., and E. N. Trifonov.** 1984. A computer algorithm for testing potential prokaryotic terminators. Nucleic Acids Res. **12:**4411–4427.
8. **Burall, L. S., J. M. Harro, X. Li, C. V. Lockatell, S. D. Himpsl, J. R. Hebel, D. E. Johnson, and H. L. Mobley.** 2004. *Proteus mirabilis* genes that contribute to pathogenesis of urinary tract infection: identification of 25 signature-tagged mutants attenuated at least 100-fold. Infect. Immun. **72:**2922–2938.
9. **Burnette-Curley, D., V. Wells, H. Viscount, C. L. Munro, J. C. Fenno, P. Fives-Taylor, and F. L. Macrina.** 1995. FimA, a major virulence factor associated with *Streptococcus parasanguis* endocarditis. Infect. Immun. **63:**4669–4674.
10. **Cain, B. D., P. J. Norton, W. Eubanks, H. S. Nick, and C. M. Allen.** 1993. Amplification of the *bacA* gene confers bacitracin resistance to *Escherichia coli*. J. Bacteriol. **175:**3784–3789.
11. **Chalker, A. F., K. A. Ingraham, R. D. Lunsford, A. P. Bryant, J. Bryant, N. G. Wallis, J. P. Broskey, S. C. Pearson, and D. J. Holmes.** 2000. The *bacA* gene, which determines bacitracin susceptibility in *Streptococcus pneumoniae* and *Staphylococcus aureus*, is also required for virulence. Microbiology **146:**1547–1553.
12. **Chenna, R., H. Sugawara, T. Koike, R. Lopez, T. J. Gibson, D. G. Higgins, and J. D. Thompson.** 2003. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res. **31:**3497–3500.
13. **Chiang, S. L., J. J. Mekalanos, and D. W. Holden.** 1999. In vivo genetic analysis of bacterial virulence. Annu. Rev. Microbiol. **53:**129–154.
14. **Coulter, S. N., W. R. Schwan, E. Y. Ng, M. H. Langhorne, H. D. Ritchie, S. Westbrock-Wadman, W. O. Hufnagle, K. R. Folger, A. S. Bayer, and C. K. Stover.** 1998. *Staphylococcus aureus* genetic loci impacting growth and survival in multiple infection environments. Mol. Microbiol. **30:**393–404.
15. **Dankert, J., J. Krijgsveld, J. van Der Werff, W. Joldersma, and S. A. Zaat.** 2001. Platelet microbicidal activity is an important defense factor against viridans streptococcal endocarditis. J. Infect. Dis. **184:**597–605.
16. **Das, S., J. C. Noe, S. Paik, and T. Kitten.** An improved arbitrary primed PCR method for rapid characterization of transposon insertion sites. J. Microbiol. Methods, in press.
17. **Douglas, C. W., J. Heath, K. K. Hampton, and F. E. Preston.** 1993. Identity of viridans streptococci isolated from cases of infective endocarditis. J. Med. Microbiol. **39:**179–182.
18. **Durack, D. T., P. B. Beeson, and R. G. Petersdorf.** 1973. Experimental bacterial endocarditis. 3. Production and progress of the disease in rabbits. Br. J. Exp. Pathol. **54:**142–151.
19. **Folmsbee, M. J., M. J. McInerney, and D. P. Nagle.** 2004. Anaerobic growth of *Bacillus mojavensis* and *Bacillus subtilis* requires deoxyribonucleosides or DNA. Appl. Environ. Microbiol. **70:**5252–5257.
20. **Ganeshkumar, N., P. M. Hannam, P. E. Kolenbrander, and B. C. McBride.** 1991. Nucleotide sequence of a gene coding for a saliva-binding protein (SsaB) from *Streptococcus sanguis* 12 and possible role of the protein in coaggregation with actinomyces. Infect. Immun. **59:**1093–1099.
21. **Gaustad, P., and L. S. Havardstein.** 1997. Competence-pheromone in *Streptococcus sanguis*. Identification of the competence gene *comC* and the competence pheromone. Adv. Exp. Med. Biol. **418:**1019–1021.
22. **Hava, D. L., and A. Camilli.** 2002. Large-scale identification of serotype 4 *Streptococcus pneumoniae* virulence factors. Mol. Microbiol. **45:**1389–1406.
23. **He, B., J. M. Smith, and H. Zalkin.** 1992. *Escherichia coli purB* gene: cloning, nucleotide sequence, and regulation by *purR*. J. Bacteriol. **174:**130–136.
24. **Hensel, M., J. E. Shea, C. Gleeson, M. D. Jones, E. Dalton, and D. W. Holden.** 1995. Simultaneous identification of bacterial virulence genes by negative selection. Science **269:**400–403.
25. **Herzberg, M. C.** 1996. Platelet-streptococcal interactions in endocarditis. Crit. Rev. Oral Biol. Med. **7:**222–236.
26. **Herzberg, M. C., G. D. MacFarlane, K. Gong, N. N. Armstrong, A. R. Witt, P. R. Erickson, and M. W. Meyer.** 1992. The platelet interactivity phenotype of *Streptococcus sanguis* influences the course of experimental endocarditis. Infect. Immun. **60:**4809–4818.
27. **Hoen, B.** 2002. Platelets and platelet inhibitors in infective endocarditis. Curr. Infect. Dis. Rep. **4:**299–303.
28. **Horton, R. M.** 1995. PCR-mediated recombination and mutagenesis. SOEing together tailor-made genes. Mol. Biotechnol. **3:**93–99.
29. **Jones, A. L., K. M. Knoll, and C. E. Rubens.** 2000. Identification of *Streptococcus agalactiae* virulence genes in the neonatal rat sepsis model using signature-tagged mutagenesis. Mol. Microbiol. **37:**1444–1455.
30. **Jordan, A., and P. Reichard.** 1998. Ribonucleotide reductases. Annu. Rev. Biochem. **67:**71–98.
31. **Kerrigan, S. W., I. Douglas, A. Wray, J. Heath, M. F. Byrne, D. Fitzgerald, and D. Cox.** 2002. A role for glycoprotein Ib in *Streptococcus sanguis*-induced platelet aggregation. Blood **100:**509–516.
32. **Kilian, M., L. Mikkelsen, and J. Henrichsen.** 1989. Taxonomic study of viridans streptococci: description of *Streptococcus gordonii* sp. nov. and emended descriptions of *Streptococcus sanguis* (White and Niven 1946), *Streptococcus oralis* (Bridge and Sneath 1982), and *Streptococcus mitis* (Andrewes and Horder 1906). Int. J. Syst. Bacteriol. **39:**471–484.
33. **Kiliç, A. O., M. C. Herzberg, M. W. Meyer, X. Zhao, and L. Tao.** 1999. Streptococcal reporter gene-fusion vector for identification of in vivo expressed genes. Plasmid **42:**67–72.
34. **Kitten, T., C. L. Munro, S. M. Michalek, and F. L. Macrina.** 2000. Genetic characterization of a *Streptococcus mutans* LraI family operon and role in virulence. Infect. Immun. **68:**4441–4451.
35. **Komano, T., R. Utsumi, and M. Kawamukai.** 1991. Functional analysis of the *fic* gene involved in regulation of cell division. Res. Microbiol. **142:**269–277.
36. **Lampe, D. J., B. J. Akerley, E. J. Rubin, J. J. Mekalanos, and H. M. Robertson.** 1999. Hyperactive transposase mutants of the *Himar1 mariner* transposon. Proc. Natl. Acad. Sci. USA **96:**11428–11433.
37. **Lampe, D. J., M. E. Churchill, and H. M. Robertson.** 1996. A purified *mariner* transposase is sufficient to mediate transposition *in vitro*. EMBO J. **15:**5470–5479.
38. **Lau, G. W., S. Haataja, M. Lonetto, S. E. Kensit, A. Marra, A. P. Bryant, D. McDevitt, D. A. Morrison, and D. W. Holden.** 2001. A functional genomic analysis of type 3 *Streptococcus pneumoniae* virulence. Mol. Microbiol. **40:**555–571.
39. **Li, Y.-H., P. C. Y. Lau, J. H. Lee, R. P. Ellen, and D. G. Cvitkovitch.** 2001. Natural genetic transformation of *Streptococcus mutans* growing in biofilms. J. Bacteriol. **183:**897–908.
40. **Loo, C. Y., D. A. Corliss, and N. Ganeshkumar.** 2000. *Streptococcus gordonii* bofilm formation: identification of genes that code for biofilm phenotypes. J. Bacteriol. **182:**1374–1382.
41. **Macrina, F. L., J. A. Tobian, K. R. Jones, R. P. Evans, and D. B. Clewell.** 1982. A cloning vector able to replicate in *Escherichia coli* and *Streptococcus sanguis*. Gene **19:**345–353.
42. **Martin, B., M. Prudhomme, G. Alloing, C. Granadel, and J. P. Claverys.** 2000. Cross-regulation of competence pheromone production and export in the early control of transformation in *Streptococcus pneumoniae*. Mol. Microbiol. **38:**867–878.
43. **Masalha, M., I. Borovok, R. Schreiber, Y. Aharonowitz, and G. Cohen.** 2001. Analysis of transcription of the *Staphylococcus aureus* aerobic class Ib and anaerobic class III ribonucleotide reductase genes in response to oxygen. J. Bacteriol. **183:**7260–7272.
44. **Mecsas, J.** 2002. Use of signature-tagged mutagenesis in pathogenesis studies. Curr. Opin. Microbiol. **5:**33–37.
45. **Mei, J. M., F. Nourbakhsh, C. W. Ford, and D. W. Holden.** 1997. Identification of *Staphylococcus aureus* virulence genes in a murine model of bacteraemia using signature-tagged mutagenesis. Mol. Microbiol. **26:**399–407.
46. **Miller, J. D., and M. N. Neely.** 2005. Large-scale screen highlights the importance of capsule for virulence in the zoonotic pathogen *Streptococcus iniae*. Infect. Immun. **73:**921–934.
47. **Moreillon, P., J. M. Entenza, P. Francioli, D. McDevitt, T. J. Foster, P. Francois, and P. Vaudaux.** 1995. Role of *Staphylococcus aureus* coagulase

and clumping factor in pathogenesis of experimental endocarditis. Infect. Immun. **63:**4738–4743.
48. **Moreillon, P., and Y. A. Que.** 2004. Infective endocarditis. Lancet **363:**139–149.
49. **Munro, C. L.** 1998. The rat model of endocarditis. Methods Cell Sci. **20:** 203–207.
50. **Munro, C. L., and F. L. Macrina.** 1993. Sucrose-derived exopolysaccharides of *Streptococcus mutans* V403 contribute to infectivity in endocarditis. Mol. Microbiol. **8:**133–142.
51. **Noe, J.** 2003. Characterization of *Streptococcus sanguis* mutants generated by signature-tagged mutagenesis. M.S. thesis. Virginia Commonwealth University, Richmond.
52. **Oetjen, J., P. Fives-Taylor, and E. H. Froeliger.** 2002. The divergently transcribed *Streptococcus parasanguis* virulence-associated *fimA* operon encoding an $Mn2^{+}$-responsive metal transporter and *pepO* encoding a zinc metallopeptidase are not coordinately regulated. Infect. Immun. **70:**5706–5714.
53. **Paik, S., A. Brown, C. L. Munro, C. N. Cornelissen, and T. Kitten.** 2003. The *sloABCR* operon of *Streptococcus mutans* encodes an Mn and Fe transport system required for endocarditis virulence and its Mn-dependent repressor. J. Bacteriol. **185:**5967–5975.
54. **Parsek, M. R., and P. K. Singh.** 2003. Bacterial biofilms: an emerging link to disease pathogenesis. Annu. Rev. Microbiol. **57:**677–701.
55. **Plummer, C., H. Wu, S. W. Kerrigan, G. Meade, D. Cox, and C. W. I. Douglas.** 2005. A serine-rich glycoprotein of *Streptococcus sanguis* mediates adhesion to platelets via GPIb. Br. J. Haematol. **129:**101–109.
56. **Polissi, A., A. Pontiggia, G. Feger, M. Altieri, H. Mottl, L. Ferrari, and D. Simon.** 1998. Large-scale identification of virulence genes from *Streptococcus pneumoniae*. Infect. Immun. **66:**5620–5629.
57. **Rice, P., I. Longden, and A. Bleasby.** 2000. EMBOSS: the European Molecular Biology Open Software Suite. Trends Genet. **16:**276–277.
58. **Roberts, R. B., A. G. Krieger, N. L. Schiller, and K. C. Gross.** 1979. Viridans streptococcal endocarditis: the role of various species, including pyridoxal-dependent streptococci. Rev. Infect. Dis. **1:**955–966.
59. **Strom, B. L., E. Abrutyn, J. A. Berlin, J. L. Kinman, R. S. Feldman, P. D. Stolley, M. E. Levison, O. M. Korzeniowski, and D. Kaye.** 1998. Dental and cardiac risk factors for infective endocarditis. A population-based, case-control study. Ann. Intern. Med. **129:**761–769.
60. **Sullam, P. M., U. Frank, M. R. Yeaman, M. G. Tauber, A. S. Bayer, and H. F. Chambers.** 1993. Effect of thrombocytopenia on the early course of streptococcal endocarditis. J. Infect. Dis. **168:**910–914.
61. **Sztukowska, M., M. Bugno, J. Potempa, J. Travis, and D. M. Kurtz, Jr.** 2002. Role of rubrerythrin in the oxidative stress response of *Porphyromonas gingivalis*. Mol. Microbiol. **44:**479–488.
62. **Tart, R. C., and I. van de Rijn.** 1991. Analysis of adherence of *Streptococcus defectivus* and endocarditis-associated streptococci to extracellular matrix. Infect. Immun. **59:**857–862.
63. **Tartof, K. D., and C. A. Hobbs.** 1987. Improved media for growing plasmid and cosmid clones. Focus **9:**12.
64. **Viola, R. E.** 2001. The central enzymes of the aspartate family of amino acid biosynthesis. Acc. Chem. Res. **34:**339–349.
65. **Voskuil, M. I., and G. H. Chambliss.** 1998. The −16 region of *Bacillus subtilis* and other gram-positive bacterial promoters. Nucleic Acids Res. **26:**3584–3590.
66. **Wells, V. D., C. L. Munro, M. C. Sulavik, D. B. Clewell, and F. L. Macrina.** 1993. Infectivity of a glucan synthesis-defective mutant of *Streptococcus gordonii* (Challis) in a rat endocarditis model. FEMS Microbiol. Lett. **112:**301–305.
67. **Zhou, T., M. Daugherty, N. V. Grishin, A. L. Osterman, and H. Zhang.** 2000. Structure and mechanism of homoserine kinase: prototype for the GHMP kinase superfamily. Struct. Fold Des. **8:**1247–1257.

Editor: J. N. Weiser